



TITLE:

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CITATION:

Minamino, Kento ...[et al]. IRF-2 regulates B-cell proliferation and antibody production through distinct mechanisms.. International immunology 2012, 24(9): 573-581

ISSUE DATE:

2012-07-05

URL:

<http://hdl.handle.net/2433/176343>

RIGHT:

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IRF-2 regulates B cell proliferation and antibody production through distinct mechanisms

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Running title: Roles of IRF-2 in B cell activation.

Key words: B cells, activation, differentiation, antibody production, interferon regulatory factor-2.

This manuscript contains, in total, 28 pages, and 5 figures

Abstract

Interferon regulatory factor (IRF)-2 is a transcription factor involved in type I interferon (IFN- α/β) signaling. It has been reported that IRF-2 deficiency results in various immune dysfunctions. However, the role of IRF-2 in B cell functions needed to be elucidated.

Unlike wild type (WT) B cells, IRF-2^{-/-} B2 cells were refractory to anti-IgM, but not LPS. Such a defect in proliferation was dependent on IFN- α/β receptor (IFNAR). Marginal zone B cells increased in the proportion relative to B2 cells in IRF-2^{-/-} mice produced IgM normally to LPS stimulation. However, IRF-2^{-/-} B2 cells were defective in IgM production in an IFNAR-independent manner, although both B cell subsets differentiated phenotypically to plasma cells at elevated efficiencies. Class switch recombination of IRF-2^{-/-} B2 cells by LPS plus IL-4 was also impaired. Their reduced IgM production was conceivably due to an inefficient upregulation of Blimp-1. Consistent with these *in vitro* observations, specific antibody production *in vivo* to a T-dependent antigen by B2 cells was severely impaired in IRF-2^{-/-} mice. However, a low, but significant, level of IgG was detected at a late time point, and this IgG exhibited comparable binding affinity to that in WT mice. Follicular helper T cell development and germinal center formation were normal. A similar tendency was observed when μ chain^{-/-} mice were reconstituted with IRF-2^{-/-} B cells.

These results revealed a multi-faceted role of IRF-2 in the function of B cells, particularly B2 cells, through regulating proliferation in an IFNAR-dependent manner and antibody production via upregulation of Blimp-1.

Introduction

Interferon regulatory factors (IRFs) have been identified as regulators of human IFN- β gene and have been shown to play a pivotal role in IFN- α/β production and its signaling in the innate immune system (1,2). In addition, they control B cell development, activation and plasma cell (PC) differentiation in the adaptive immune responses (3). IRF-4 and IRF-8 control immunoglobulin light chain gene rearrangement in the differentiation of B cells (4,5). IRF-8 also regulates pre-BCR expression (6) and differentiation to B2 and MZB cells in the spleen (7). Furthermore, IRF-4 and IRF-5 directly induce *Prdm1* (coding Blimp-1) expression and initiate the PC development in the germinal centers (GC) (8,9).

In the IRF family, IRF-2 was originally identified as an antagonist of IRF-1 (2,10). Further analyses revealed that IRF-2 competes with IFN-stimulated gene factor (ISGF) 3, hetero-trimer of STAT-1, STAT-2 and IRF-9, to bind ISGF-binding motif in promoters of IFN- α/β responsive genes, resulting in the suppression of IFN- α/β signals *via* IFNAR (2,11). This antagonistic function is important in order to attenuate IFN- α/β receptor (IFNAR)-dependent signals evoked by spontaneously produced IFN- α/β (12-15). In addition, IRF-2 binds to IFN- β and CIITA-pIV promoters and regulates their functions by competing with IRF-1 and Blimp-1 in cellular responses (16-18).

Regarding immune cell development, IRF-2^{-/-} mice have been shown to lack CD4⁺ dendritic cells (13,14) and NK cells (19) with spontaneous expansion of basophils (20). A previous study also shows that IRF-2-deficiency suppresses hematopoiesis and B lymphopoiesis in the bone marrow (21). However, detailed analyses of IRF-2 in mature B cell functions remains to be elucidated for proliferative and antibody (Ab) responses to thymus-dependent (TD) and thymus-independent type II (TI-2) antigens.

Therefore, we aimed to investigate this issue. Our results show that IRF-2 affects

mainly B2, but not marginal zone B (MZB), cell functions in terms of anti-IgM-induced proliferation by regulating IFNAR-mediated signals and Ab production by elevating Blimp-1 expression.

Materials and Methods

Mice and immunization

C57BL/6 were purchased from Japan SLC (Shizuoka, Japan). The generation of IRF-2^{-/-}, IFNAR^{-/-} and IRF-2^{-/-}IFNAR^{-/-} mice has been described previously (14). μ chain^{-/-} mice (μ MT) were kindly provided by Dr. Tomohiro Kurosaki (RCBI, Yokohama, Japan) and 5×10^6 to 1×10^7 B cells were transferred into these mice 1 d before immunization. All mice were maintained under specific pathogen-free conditions and used at 6 - 10 weeks of age. Mice were immunized with 20 μ g TNP-OVA in CFA (BD Biosciences, San Diego, CA, USA) or 20 μ g trinitrophenyl (TNP)-Ficoll in PBS, and sera were collected at 0, 7 and 14 d. All experiments were performed according to the institutional Guidelines for Animal Use and Experimentation of Kyoto University.

Antibodies and reagents

FITC-anti-IgM was purchased from Roche Diagnostics (Hague Road, IN, USA). FITC-anti-CD4, PE-anti-CD3 ϵ and PE-anti-CD21 were obtained from BD Biosciences. FITC-anti-CD23, PE-Cy7-anti-B220, biotin-anti-CXCR5 and biotin-anti-mouse IgM were from eBioscience (San Diego, CA, USA). F(ab')₂ goat anti-mouse IgM, Cy5-mouse anti-rat IgG, Streptavidin-Cy3, HRP-anti-rabbit IgG and AP-anti-mouse IgM and IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-IRF-4 and HRP-anti-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Streptavidin-Texas Red was from Molecular Probes (Eugene, OR, USA). TNP-Ficoll and TNP-BSA were from Biosearch Technologies (Novato, CA, USA). Biotin-PNA was purchased from Wako Inc. (Tokyo, Japan). Anti-CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD16/32 (2.4G2), CD45R (B220), MHC class II (TIB120) and Gr-1 (RB6-8C5)

monoclonal antibodies (mAbs) were used as hybridoma supernatants. Purified anti-CD40 (FGK45.2) and anti-CD43 (S7) mAbs were kind gifts from Dr. R. M. Steinman (Rockefeller University, NY, USA) and Dr. Koichi Ikuta (Kyoto University, Kyoto, Japan), respectively.

Cell preparations, flowcytometry and immunohistochemistry

Spleen and subcutaneous lymph node (LN) B cells were negatively enriched by anti-CD4, CD8, CD11b, CD43 and Gr-1 mAbs in combination with sheep anti-rat IgG Dynabeads (Dyna, Lake Success, NY, USA). The resulting cells consisted of >94% B220⁺ cells. In some experiments, purified B cells stained with anti-CD21, anti-CD23 and anti-B220 were sorted for B2 cells as B220⁺CD23⁺CD21^{low} and MZB cells as B220⁺CD23⁻CD21^{high} by FACS Aria (BD Biosciences). Preparation of CD4⁺ T cells was described previously (22).

Cells from spleen were stained with the mAbs indicated in the figure legends and acquired using FACSCalibur (BD Biosciences). Data were analyzed by FlowJo software (Tree Star Inc., San Carlos, CA, USA). Immunohistochemistry was performed as described previously (22) using the mAbs indicated in the figure legends.

In vitro culture and antibody production

Purified B cells were cultured in 100 μ l of RPMI1640 supplemented with heat-inactivated 5% FCS, 50 μ M 2-mercaptoethanol (culture medium) at 1.5×10^5 cells/well in 96-well flat-bottomed plates, and stimulated with either 100 U/ml IL-4 (eBioscience), 15 μ g/ml F(ab')₂ goat anti-mouse IgM Ab, 10 μ g/ml agonistic anti-CD40 mAb, 10 μ g/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) alone or their combination. Proliferation was assessed by [³H]-thymidine (TdR) incorporation between 40-48 h of culture. For Ab production, 1.5×10^5 B cells were stimulated with 10 μ g/ml LPS in the presence or absence of 1,000 U/ml IL-4 for 3 d and the amounts of IgM and IgG1 in the culture supernatant were assessed by ELISA

using plates coated with anti-mouse Ig Ab.

Anti-TNP Abs in serum were also assayed by ELISA using plates coated with TNP-BSA with serially diluted serum, followed by goat-anti-mouse IgM or IgG. Results are shown as an index, which was calculated by dividing the half-max optical density value of the serum dilution at 7 d and 14 d by that of 0 d. Affinity of Ab was calculated by dividing the half-max optical density value of the serum dilution to anti-TNP₅-BSA by that to anti-TNP₁₉-BSA at 7 d and 14 d.

Detection of gene expression

Quantitative RT-PCR was performed to detect gene expressions. Briefly, total RNA was extracted from B or T cells with TRIzol[®] (Invitrogen, Carlsbad, CA, USA). In some experiments, cDNA was generated with SuperScript[®] III (Invitrogen). Quantitative PCR was performed with QuantiTect SYBR Green PCR Kits (Qiagen, Valencia, CA, USA) or SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Data were collected by Rotor-gene 2000 (Qiagen), and the relative expression was quantified by comparative Ct assay. The following primers were used:

Pax5: 5'-GTCCCCCAGTGCCGACACCA-3' and 5'-AGCACCTCCAGCTGCTGCTG-3',
Blimp1: 5'-GCGCTGACGGGGTACTTCTG-3' and
5'-CACCGATGAGGGGTCCAAAGCG-3', IRF-4: 5'-TCTGCTGAAGCCTTGGCGCTC-3'
and 5'-TGTCCGTGGGAGATCCGGCA-3', Bcl-6: 5'-ACGCTGCAGACGCACAGTGA-3'
and 5'-GGCCGATTGAACTGCGCTCCA-3', Bach2:
5'-AGACTCGAAGCCAGGACAGGACC-3' and
5'-CCCAGGAGGATGTTGGCACAGTG-3', IL-21:
5'-GGGCCAGATCGCTCCTGATTAG-3' and
5'-AAACAGGCAAAAGCTGCATGCTCAC-3', GAPDH:

5'-AGACGGCCGCATCTTCTTGTGC-3' and 5'-GCCACTGCAAATGGCAGCCC-3'.

For Western blot analysis, B cell lysates were prepared before or after LPS stimulation with lysis buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 25 mM CaCl₂]. Samples were separated by 12% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), probed with anti-IRF-4 Ab and HRP-anti-Rab IgG or HRP-anti-actin, and then visualized with LumiGLO chemiluminescent substrate (Cell Signaling Technology, Beverly, MA, USA).

Transduction of IRF-2 cDNA to LPS-activated B2 B cells.

IRF-2 cDNA was cloned into MSCV-based retroviral vector (a gift from Dr. Tomohiro Kurosaki) containing EGFP downstream of the internal ribosomal entry site. The Plat-E packaging cells were transiently transfected with retroviral vector with the Lipofectamine 2000 reagent (Invitrogen) to produce viral supernatant. Purified B2 B cells from IRF-2^{-/-} or WT mice were stimulated with LPS for 1 d and transduced with retrovirus in the presence of 10 µg/ml polybrene. Two days after infection, EGFP⁺ cells were sorted and cultured at 3.5 to 5 × 10⁴ cells/well without stimuli. The supernatant for ELISA and total RNA for RT-PCR were collected 3 d later.

Statistical analysis

Data are expressed as the mean ± SD of triplicate cultures or the sum of individual experiments. Statistical significances were determined by the unpaired Student's *t*-test or one-way ANOVA. Differences were considered to be significant for *p* values < 0.05. All experiments were performed at least 3 times and representative results are shown.

Results

Reduced proliferation of IRF-2^{-/-} B cells in response to BCR-mediated stimulation in an IFNAR-dependent manner

We first examined B cell proliferative responses to various stimuli *in vitro*. Although B cells from both IRF-2^{-/-} and WT mice responded comparably to LPS, IRF-2^{-/-} B cells were severely impaired in their response to BCR-mediated stimulation (Fig. 1A). However, the IRF-2^{-/-} B cells appeared to be better responders than WT B cells for agonistic anti-CD40 mAb.

Since IRF-2 is involved in IFNAR-mediated signaling, the proliferative responsiveness of IRF-2^{-/-} spleen B cells to anti-IgM was compared with that of IRF-2^{-/-}IFNAR^{-/-} B cells. As shown in Fig. 1B, the reduced B cell proliferation in IRF-2^{-/-} mice was restored in IRF-2^{-/-}IFNAR^{-/-} mice to nearly comparable levels in WT and IFNAR^{-/-} mice, implying that IRF-2 counteracts the IFNAR-dependent signals that inhibits BCR-induced proliferation.

Decreased IgM production by LN, but not spleen, B cells in response to LPS

LPS is a most potent mitogen for B cells to induce the proliferation and IgM production. Since LPS evoked comparable proliferation between WT and IRF-2^{-/-} B cells, we next examined the IgM production by spleen and LN B cells from IRF-2^{-/-}, IFNAR^{-/-}, IRF-2^{-/-}IFNAR^{-/-} and WT mice. Ab production of spleen B cells appeared to be normal in all strains of mice (Fig. 1C, *left panel*), whereas LN B cells, which consist mainly of B2 cells, showed significantly decreased IgM production not only in IRF-2^{-/-} but also in IRF-2^{-/-}IFNAR^{-/-} mice (Fig. 1C, *right panel*). These results indicate that, unlike the proliferation, the reduced Ab-producing ability of IRF-2^{-/-} B2 cells is not mediated by

IFNAR-dependent signals.

Skewed B cell development to MZB rather than to B2 in IRF-2^{-/-} mice

It is well known that spleen B cells are comprised of B2 and MZB cells (23). A previous study has suggested that, in contrast to B2 cells *in vitro*, BCR stimulation leads to the apoptosis of MZB cells, and LPS treatment induces rapid and robust levels of proliferation and differentiation to Ab forming cells (24). Taking the results shown in Fig. 1C into account, it is plausible that the spleen B cell composition in IRF-2^{-/-} mice is skewed to MZB cells. To examine this possibility, B cell populations were compared between IRF-2^{-/-} and WT mice by gating on B220⁺ cells (Fig. 2A, *left panels*). MZB cells (CD21⁺CD23⁻) were a minor population in WT mice. In contrast, those in IRF-2^{-/-} mice were markedly increased. In addition, the number of B2 cells in the IRF-2^{-/-} mice was reduced to half that in the WT mice (Fig. 2A, *right panels*). However, the number of B cells in the LN was not significantly reduced in IRF-2^{-/-} mice (data not shown). Increased MZB cells were also confirmed by their phenotype of IgM⁺IgD⁻ as revealed in the immunohistochemical staining with anti-IgM and IgD (Fig. 2B, *left panels*), followed by measuring the thickness of MZ (Fig. 2B, *right panel*). Similar results were obtained by the staining of MZ macrophages with ER-TR9 mAb (data not shown).

Defect in B2, but not MZB, cell responsiveness in IRF-2^{-/-} mice

We then re-examined proliferative responsiveness of B2 and MZB cells separately (Fig.3A). The results showed that IRF-2^{-/-} B2 cells were incapable of proliferating in response to anti-IgM, but responded to LPS more vigorously than WT B2 cells (Fig. 3A, *left panel*). As reported (25), both IRF-2^{-/-} and WT MZB cells were unresponsive to BCR-dependent signaling but responded equally to LPS stimulation (Fig. 3A, *right panel*). Like B2 cells,

IRF-2^{-/-} MZB cells looked to be more reactive to agonistic anti-CD40 mAb than WT MZB cells. However, the severe reduction of BCR-induced proliferation in IRF-2^{-/-} B2 cells was partially restored by either IL-4 or anti-CD40 alone. When both IL-4 and anti-CD40 were added, IRF-2^{-/-} B2 cells equally responded to WT B2 cells (Fig. 3B).

In regard to Ab production, the B2, but not MZB, cell response to LPS was significantly impaired in IRF-2^{-/-} (Fig. 3C), although there was good proliferation (Fig. 3A). In contrast to Ab production, the differentiation to PC in phenotypes was unexpectedly augmented in IRF-2^{-/-} B2 cells (Fig. 3D). This accelerated differentiation was also seen in IRF-2^{-/-} MZB cells. As to class switch recombination (CSR) from IgM to IgG1, IRF-2^{-/-} B2 cells were less efficient and produced lower amounts of IgG1 than WT B2 cells when stimulated with LPS and IL-4 (Figs. 3E and 3F). These results show that the unresponsiveness of IRF-2^{-/-} splenic B cells can be ascribed to the defect in the B2, but not MZB, cells, and indicate that IRF-2 expression is required for the BCR-mediated proliferation and LPS-induced differentiation into Ab-producing PC as well as CSR of B2 cells.

Inefficient upregulation of Blimp-1 by LPS in IRF-2^{-/-} B2 cells

To explore the stage affected by IRF-2 deficiency in B cell differentiation into PC, we analyzed the mRNA expression of *Pax5*, *Prdm1* (coding Blimp-1), *Irf4*, *Bcl6* and *Bach2* in IRF-2^{-/-} B2 cells. Prior to LPS stimulation, LN B cells from IRF-2^{-/-} expressed comparable levels of *Pax5*, *Prdm1*, *Irf4* and *Bach2*, but partial reduction of *Bcl6* (Fig. 4A, *top panels*). After stimulation with LPS for 3 d, *Pax5*, *Irf4*, *Bcl6* and *Bach2* expression was downregulated in IRF-2^{-/-} B2 as well as in WT B2 cells. Notably, upregulation of *Prdm1*, observed readily in WT B2 cells, was severely impaired in IRF-2^{-/-} B2 cells. Interestingly, both IRF-2^{-/-} and WT MZB cells, which were able to differentiate into PC (see Fig.3D), downregulated *Pax5*, *Bcl6* and *Bach2*, and upregulated *Irf4* and *Prdm1* mRNA (Fig. 4A, *bottom panels*). Furthermore,

stimulated B2 cells increased IRF-4 protein (Fig. 4B), although its mRNA was decreased in both IRF-2^{-/-} and WT B2 cells (Fig. 4A).

To confirm the role for IRF-2 in Blimp-1 induction and Ab production, we retrovirally transduced IRF-2 cDNA into IRF-2^{-/-} B2 cells. Complementation of IRF-2 indeed augmented Blimp-1 expression (Fig. 4C) along with the elevation of Ab production (Fig. 4D). When WT B2 cells were transduced with IRF-2 cDNA, Blimp-1 expression was further upregulated (Fig. 4E), but IgM production was not affected (Fig. 4F). Perhaps the amounts of Blimp-1 in WT B2 cells were already sufficient to induce IgM production. These results suggest that IRF-2 is deeply involved in *Prdm1* upregulation and Ab production in B2 cells.

Reduced Ab production in response to TD, but not TI-2 antigen, in IRF-2^{-/-} mice

To investigate the role of IRF-2 in B cell function *in vivo*, we examined the Ab producing ability against TNP-Ficoll, a TI-2 antigen, and TNP-OVA/CFA, a TD antigen. After a single injection of antigens, TNP-specific IgM and IgG in the sera of IRF-2^{-/-} mice were compared at 7 and 14 d with those of WT mice. In response to TNP-Ficoll, IRF-2^{-/-} and WT mice produced comparable levels of TNP-specific IgM and IgG (Fig. 5A). In sharp contrast, Ab production, particularly that of IgM, to TD antigen was severely impaired at 7 and 14 d in the IRF-2^{-/-} mice (Fig. 5B). Although the IgG production was severely reduced at 7 d, it increased to significant, but still markedly low, levels as compared with those in WT mice at 14 d. Of note, the affinity maturation at 14 d was comparable between IRF-2^{-/-} and WT mice (Fig. 5C).

Since high-affinity IgG Ab in response to TD antigen is produced by PCs generated in the germinal center (GC) (26), GC formation was also examined in IRF-2^{-/-} and WT mice. Immunohistochemical staining of spleen sections at 14 d with PNA and anti-IgD demonstrated that GC formation in IRF-2^{-/-} mice was not severely impaired, although PNA⁺

cells looked slightly sparse (Fig. 5D). However, GC B cells identified as PNA⁺Fas⁺ were not significantly reduced in IRF-2^{-/-} mice compared with WT mice (Fig. 5E). In addition, there was no significant difference in apoptotic GC B cells between IRF-2^{-/-} and WT mice (data not shown). Consistent with GC formation and normal GC B cell development, comparable numbers of follicular helper T (Tfh) cells were present in the IRF-2^{-/-} spleen at 14 d (Fig. 5F). Moreover, IL-21 and Bcl-6 mRNA expression in CD4⁺ T cells was also comparable at 10 d between IRF-2^{-/-} and WT (Fig. 5G).

To verify that the profoundly reduced Ab-producing response to TD antigen in the IRF-2^{-/-} mice could be attributed to B cell intrinsic defects, but not CD4⁺ T cell function, we transferred purified IRF-2^{-/-} or WT B cells to μ MT mice and immunized these mice with TNP-OVA/CFA (Fig. 5H). Neither IgM at 7 d nor IgG levels at 7 d or 14 d were fully restored in IRF-2^{-/-} B cell-reconstituted mice, indicating that the impaired Ab response in the IRF-2^{-/-} mice is due to intrinsic B cell defects. It should be noted that no measurable amounts of IgM were detected at 14 d in this experimental design (data not shown). These data suggest that IRF-2 is necessary for an optimal Ab response, likely at the level of antibody production, to TD antigen by B2 cells *in vivo*.

Discussion

The present study demonstrates that IRF-2 is involved in B2, but not MZB, cell proliferation and differentiation into Ab-producing PC induced by *in vitro* stimulation with anti-IgM and LPS, respectively. Importantly, IRF-2 contributes to these two B2 cell functions through different mechanisms. As in *in vitro* assays, the IRF-2^{-/-} mice responded normally to TI-2 Ag, but dramatically reduced IgM as well as IgG production in response to TD Ag *in vivo*.

With respect to B cell proliferation, IRF-2 regulates BCR- but not LPS-induced signals by antagonizing IFNAR-mediated signaling, indicating that IFNAR signaling could act as an endogenous negative regulator of BCR-stimulated B cell proliferation. We did not directly examine the proliferative responses of purified B2 cells from IRF-2^{-/-}IFNAR^{-/-} mice. However, since MZB cells did not respond to anti-IgM stimulation irrespective of their genotype, anti-IgM-induced proliferation by IRF-2^{-/-}IFNAR^{-/-} splenic B cells, which is as strong as that by WT B cells, appears to be attributable to the restoration of B2 cell responses, indicating that IFN- α/β are an intrinsic attenuator of B2 cell proliferation. In support of this possibility, BCR-mediated proliferation of WT B2 cells was reduced when large amounts of exogenous IFN- α were added (data not shown). However, previous studies have shown that IFN- α/β positively controls BCR-mediated activation of immature and mature B cells and rescues them from cell death (27-29). Reconciling these apparently contradicting observations, we infer that IRF-2 sets a threshold for IFNAR signals, preventing too strong and/or chronic IFN- α/β signals from inhibiting B2 cell proliferation.

Regarding the apparently skewed B cell development to MZB rather than to B2 cells in IRF-2^{-/-} mice, the mechanisms involved are not clear. However, there is a similar B cell composition in IRF-2^{-/-}IFNAR^{-/-} and IRF-2^{-/-} mice (data not shown). Therefore, an endogenous

IFN- α/β , or IFNAR-mediated signal seems not to be directly involved in spleen B cell development and survival *in vivo*. On the other hand, it has been reported that pre-B and immature B cell numbers are severely reduced in IRF-2^{-/-} animals with a mixed background of C57BL/6 and 129 strains (21), and we also observed that apoptosis of differentiating B cells at the stage of pro- and pre-B cells in the bone marrow looked to be a little higher in IRF-2^{-/-} than WT mice (data not shown), implying that IRF-2 plays a role in controlling B cell development in the early stages.

IRF-2 in B2, but not MZB, cells is required for *in vitro* IgM production upon LPS stimulation. Blimp-1 is a master regulator of PC differentiation and is necessary for Ab secretion (30). In accordance with decreased IgM production by B2 cells, upregulation of Blimp-1 is impaired in IRF-2^{-/-} LN B cells, which consist predominantly of B2 cells. Unlike B2 cells, IRF-2^{-/-} MZB cells produced nearly comparable levels of IgM to WT MZB cells and upregulated Blimp-1 expression as efficiently as WT MZB cells. However, other transcriptional factors, such as Pax5, Bcl-6 and Bach2 including IRF-4, were similarly regulated in IRF-2^{-/-} as in WT B2 cells.

Other IRF family members, such as IRF-4 and IRF-5, have been shown to bind the *Prdm1* promoter and to upregulate Blimp-1 expression (8,9). At this time point, there is no direct evidence for the direct binding of IRF-2 to the *Prdm1* promoter. The impairment of Blimp-1 upregulation was also observed in IRF-2^{-/-}IFNAR^{-/-} B2 cells (data not shown). Furthermore, enforced expression of IRF-2 in IRF-2^{-/-} B2 cells upregulated Blimp-1 expression, indicating that IRF-2 is required for the induction of Blimp-1, acting on an as-yet unidentified signaling pathway other than those mediated by IFNAR. This IFNAR-independent IRF-2 function for Blimp-1 upregulation is also involved in LPS-induced IgM production by B2 cells, since LN B cells from IRF-2^{-/-}IFNAR^{-/-} mice were as inefficient as IRF-2^{-/-} mice in IgM production, IgM production by LPS being restored by transduction of

IRF-2 gene into IRF-2^{-/-} B2 B cells.

Contrary to IgM production, IRF-2^{-/-} B2 cells differentiated to PC-like phenotypic cells expressing CD138. Blimp-1 and IRF-2 have similar binding affinities for functionally important regulatory sites containing the sequence GAAG (16-18). Therefore, the accelerated differentiation to PC-like cells may be attributed to the loss of a competitor, leading to the efficient binding of a small amount of Blimp-1 in IRF-2^{-/-} B2 cells to support PC-like differentiation, yet not sufficient for antibody production. This possibility may be supported by the observation that much vigorous PC differentiation was detected in IRF-2^{-/-} MZB cells, as opposed to what takes place in WT mice, even though both expressed comparable levels of Blimp-1.

Recently, it has been reported that spontaneous secretion of low levels of IgM by peritoneal B1 cells relies on an IRF4-independent non-classical pathway, whereas that by spleen B1 cells does rely on an IRF-4-dependent pathway (31). Therefore, the requirements for transcription factors for Ab-production may be distinct in different subsets of B cells. In this regard, the different influence of IRF-2 deficiency on Blimp-1 upregulation and IgM production by LPS in MZB and B2 cells seems to constitute another example of such a different requirement of transcription factors in different B cell subsets.

In humoral immune responses, B2 cells and MZB cells preferentially respond to TD and TI-2 antigens, respectively (25). As in *in vitro* studies, the IgM response to TD, but not TI-2, antigen was severely impaired in IRF-2^{-/-} mice at both 7 d and 14 d after immunization. Unlike IgM production, fairly low but significant levels of IgG production was detected at 14 d, although only an extremely low IgG production was detected at 7 d. Such reduced Ab responses at 7 d and 14 d were also observed when μ MT mice were reconstituted with IRF-2^{-/-} B cells, indicating that IRF-2 plays a role in B cell Ab production *in vivo*. We observed GC formation, and detected the presence of comparable numbers of GC B cells and

Tfh cells in the IRF-2^{-/-} spleen. Furthermore, the relative binding activity of anti-TNP IgG is nearly equal between IRF-2^{-/-} and WT mice. In addition, IRF-2^{-/-} B2 cells stimulated with anti-IgM normally proliferate in the presence of IL-4 and agonistic anti-CD40, but produced less IgG1 in response to LPS + IL-4, being due to the inefficient CSR. Therefore, it seems that once Tfh is induced, IRF-2^{-/-} B cells get sufficient stimulation signals to develop and sustain GCs *in vivo* but are able to produce only marginal levels of IgG.

It has been shown that IRF-2^{-/-} mice can produce comparable levels of neutralizing IgM and IgG Abs to VSV (21). As IgM production to VSV is T cell-independent (32), our result in immunization with TI-2 antigen is consistent with this previous report. However, in contrast to our observations, the VSV-specific IgG response, which depends on CD4⁺ helper T cells (33), was reported to be normal in IRF-2^{-/-} mice. The reason for such a discrepancy is not clear, but we assume that adjuvant activities of VSV, the virus, and CFA containing mycobacteria may contribute to the Ab-producing adaptive immune responses.

Since antigen-specific IgM production by B2 cells is a key step in the production of IgG against TD antigen, the reduced IgG production to TNP-OVA/CFA in IRF-2^{-/-} mice may be attributable to a severely impaired extra-follicular IgM response at the earlier time points. Given that IgM responses failed to be detected in IRF-2^{-/-} mice even on 14 d, when a considerable IgG response was observed, IRF-2 seems to be essential for extra-follicular production of IgM and important, but not mandatory, for later IgG production. Related to this issue, extra-follicular T cell development has been reported to be crucial to extra-follicular Ab responses (34,35). Since IRF-2 was reported to affect the differentiation of helper T cell subsets (20), IRF-2 may also contribute to the differentiation and function of extra-follicular helper T cells, but not Tfh cells, in addition to B2 cell activation. This possibility should be examined in future experiments.

In conclusion, our current findings revealed previously unrecognized roles of IRF-2

in B cell activation, particularly that of B2 cells, through regulation of IFNAR signals and Blimp-1 expression. Since IRF-2 deficiency affected the function and development of a wide array of immune cells, including dendritic cells, NK cells and basophils, future studies of the mechanisms for IRF-2 actions in B cells would help us understand those for other immunocytes and possibly help uncover a common regulatory principle for multiple cells in the immune system.

Abbreviation

antibody, Ab; type I interferon, class switch recombination, CSR; IFN- α/β ; type I interferon receptor, IFNAR; interferon regulatory factor, IRF; lymph node, LN; lipopolysaccharide, LPS; monoclonal antibody, mAb; marginal zone, MZ; marginal zone B, MZB; plasma cells, PC; thymidine, TdR; thymus dependent, TD; thymus independent, TI; wild type, WT.

Funding

This work was supported in part by the following grants: Core Research for Evolutional Science and Technology, and the Japan Science and Grant-in-Aid for Scientific Research (20390109 to KI) from the Ministry of Education, Science, Sports and Culture of Japan. KM and KN were also supported with Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

Acknowledgments

We acknowledge Dr. R. M. Steinman (Rockefeller University, NY, USA), Dr. Koichi Ikuta (Kyoto University, Kyoto, Japan) and Dr. Tomohiro Kurosaki (RCBI, Yokohama, Japan) for providing purified anti-CD40 (FGK45.2) mAb, anti-CD43 (S7) mAb and, μ MT mice and retroviral vector, respectively.

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Figure legends

Fig. 1. Differential regulation of BCR-induced proliferation and Ab production by IRF-2^{-/-} B cells.

(A) Purified spleen B cells from IRF-2^{-/-} or WT mice were stimulated with various stimuli and proliferation was assessed by [³H]-TdR incorporation between 40-48 h of culture. (B) Purified spleen B cells from IFNAR^{-/-} and IRF-2^{-/-}IFNAR^{-/-} as well as IRF-2^{-/-} and WT mice were stimulated with anti-IgM, and proliferation was then assessed as in (A). (C) Purified splenic (*left panel*) or LN (*right panel*) B cells from WT, IRF-2^{-/-}, IFNAR^{-/-}, and IRF-2^{-/-} IFNAR^{-/-} mice were stimulated with LPS for 3 d. Amounts of IgM in culture supernatant were determined by ELISA. Data are shown as the mean ± SD of triplicate cultures and are representative of 4 independent experiments. Statistical significance was calculated by unpaired Student's *t*-test in A, or one-way ANOVA in B and C (** *p* < 0.01).

Fig. 2. IRF-2 regulates proliferation and Ab production in B2, but not MZB, cells.

(A) Spleen cells were stained with anti-B220, CD21 and CD23 and analyzed by gating on B220⁺ cells (*left panels*). Numbers in the figures show the percentages of the respective B cell populations in B220⁺ cells. Cell numbers of each subset are depicted in the *right panel*. Results are the mean ± SD of 12 mice. ** *p* < 0.01 (*t*-test) (B) Spleen sections were stained with anti-IgM (green) and IgD (blue) (*left panels*). The thickness of MZ (IgM⁺IgD⁻) is shown (*right panel*). MZ is represented between white lines in the pictures. Distances are measured by placing perpendicular lines at 50 μm intervals to the white line adjacent to white pulp. Horizontal bars represent the mean. Data are representative of 3 independent experiments. ** *p* < 0.01 (*t*-test)

Fig. 3. Defects of IRF-2^{-/-} B2 cells in proliferation, Ab production and CSR.

(A) Purified B2 and MZB cells from IRF-2^{-/-} and WT mice were cultured with anti-IgM, anti-CD40 and LPS. Proliferation was assessed as in Fig. 1A. Data are shown as the mean \pm SD of triplicate cultures and are representative of 3 independent experiments. ** $p < 0.01$ (t -test). (B) Purified B2 cells from IRF-2^{-/-} and WT mice were cultured either alone or with anti-IgM, anti-CD40 or IL-4, or the combinations indicated. Proliferation was assessed as in Fig. 1A. (C) Purified B2 and MZB cells from IRF-2^{-/-} and WT mice were stimulated with LPS for 3 d and the amounts of IgM in supernatants were analyzed by ELISA. (D) Stimulated cells in (C) were stained with anti-B220 and anti-CD138. Numbers in figures show the percentage of B220^{low}CD138⁺ cells. (E) B2 from IRF-2^{-/-} and WT mice were stimulated with LPS in the presence or absence of IL-4 for 3 d, followed by intracellular staining with anti-IgG1 after cell surface staining with anti-B220. Numbers in figure show the percentage of IgG1⁺ cells (*left panels*). Percentages of IgG1⁺ cells are depicted in the *right panel*. Data are shown as the mean \pm SD of triplicate cultures and are representative of 3 independent experiments. (F) The amounts of IgG1 in supernatant of (E) were assessed by ELISA. Data are shown as the mean \pm SD of triplicate cultures and are representative of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ (t -test)

Fig. 4. Impaired upregulation of *Prdm1* in IRF-2^{-/-} LN B cells after LPS stimulation and rescue of the reduced IRF-2^{-/-} B2 responsiveness by IRF-2 transduction.

(A) Total RNA was extracted from LN B (*top panels*) and MZB (*bottom panels*) cells immediately after preparation (d0) or LPS stimulation for 3 d (d3). mRNA expression of *Pax5*, *Prdm1*, *Irf4*, *Bcl6* and *Bach2* was assessed by quantitative RT-PCR. Data are shown as the mean \pm SD of triplicate cultures and are representative of 3 independent experiments. ** $p < 0.01$ (t -test). (B) Expression of IRF-4 protein was determined by Western blot analysis before

and after LPS stimulation as in (A). (C) IRF-2 gene containing EGFP was retrovirally transduced into IRF-2^{-/-} B2 cells, and upregulations of *prdm1* and *irf2* in EGFP⁺ cells were determined by quantitative RT-PCR 3 d later. (D) Amount of IgM in culture supernatant of (C) was measured by ELISA. (E) As in (C), WT B2 cells were subjected to the assay. (F). As in (D), the amount of IgM from WT B2 cells that were transduced with IRF-2 gene was also determined. Data from (C-F) are shown as the mean \pm SD of triplicate cultures and are representative of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ (t -test).

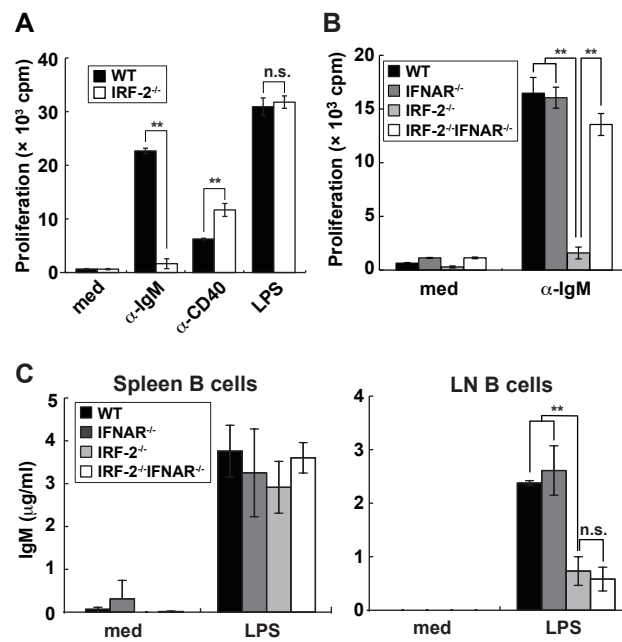
Fig. 5. Reduced antibody production in response to TD, but not TI, antigen in IRF-2^{-/-} mice.

(A, B) IRF-2^{-/-} and WT mice were immunized *ip* with TNP-Ficoll (A) or TNP-OVA/CFA (B). Sera were collected before and after 7 and 14 d of immunization and analyzed for TNP-specific IgM (*left panels*) and IgG (*right panels*) production. Results are shown as an index, calculated by dividing the half-max O.D. value of serum dilution at 7 d and 14 d by that of 0 d. Circles indicate individual mice and horizontal bars represent the mean. (C) Affinity of anti-TNP Ab is shown as the relative binding, which was calculated by dividing half-maximum serum dilution to TNP₅-BSA by that to TNP₁₉-BSA with ELISA. (D) Cryosections of spleen from IRF-2^{-/-} and WT mice at 14 d after TNP-OVA/CFA immunization were fixed and stained with anti-IgD (green) and PNA (red). Arrows indicate GCs. (E) GC B cells in spleen 14 d after stimulation as in (B) were stained with anti-B220, PNA and Fas and analyzed by gating on B220⁺ cells (*left panels*). Values are the percent of B220⁺PNA⁺Fas⁺ cells. The numbers of GC B cells were calculated (*right panel*). Bar graphs show the mean \pm SD of 6 mice. (F) Tfh cells in spleen were calculated by the staining with anti-CD3 ϵ , CD4 and CXCR5 as in (E). (G) Expressions of IL-21 and Bcl-6 were analyzed by quantitative RT-PCR in purified CD4⁺ T cells 10 d after the immunization. (H) Anti-TNP IgM and IgG production was determined 7 and 14 d after immunization as (B) in μ MT mouse

that had received purified B cells from WT and IRF-2^{-/-} mice 1 d before. To adjust the proportion of B2 and MZB cells in IRF-2^{-/-} to that in WT mice, IRF-2^{-/-} LN B cells were added to IRF-2^{-/-} bulk spleen B cells. * $p < 0.05$, ** $p < 0.01$ (t -test).

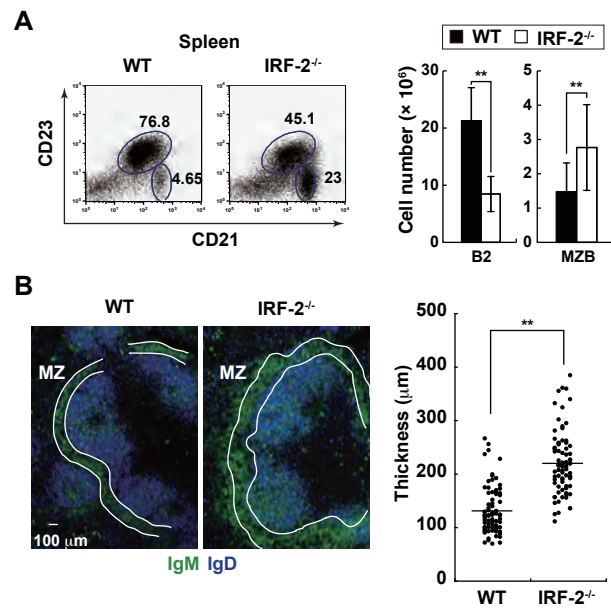
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Figure 1.



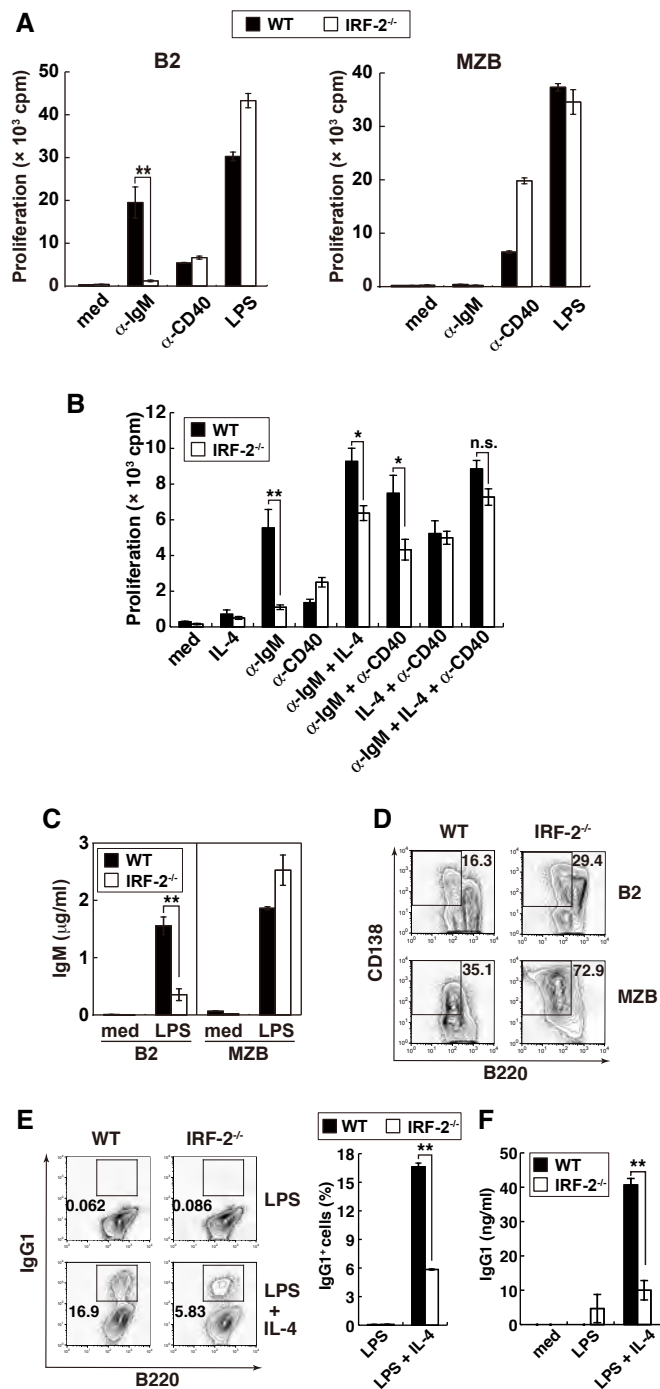
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Figure 2



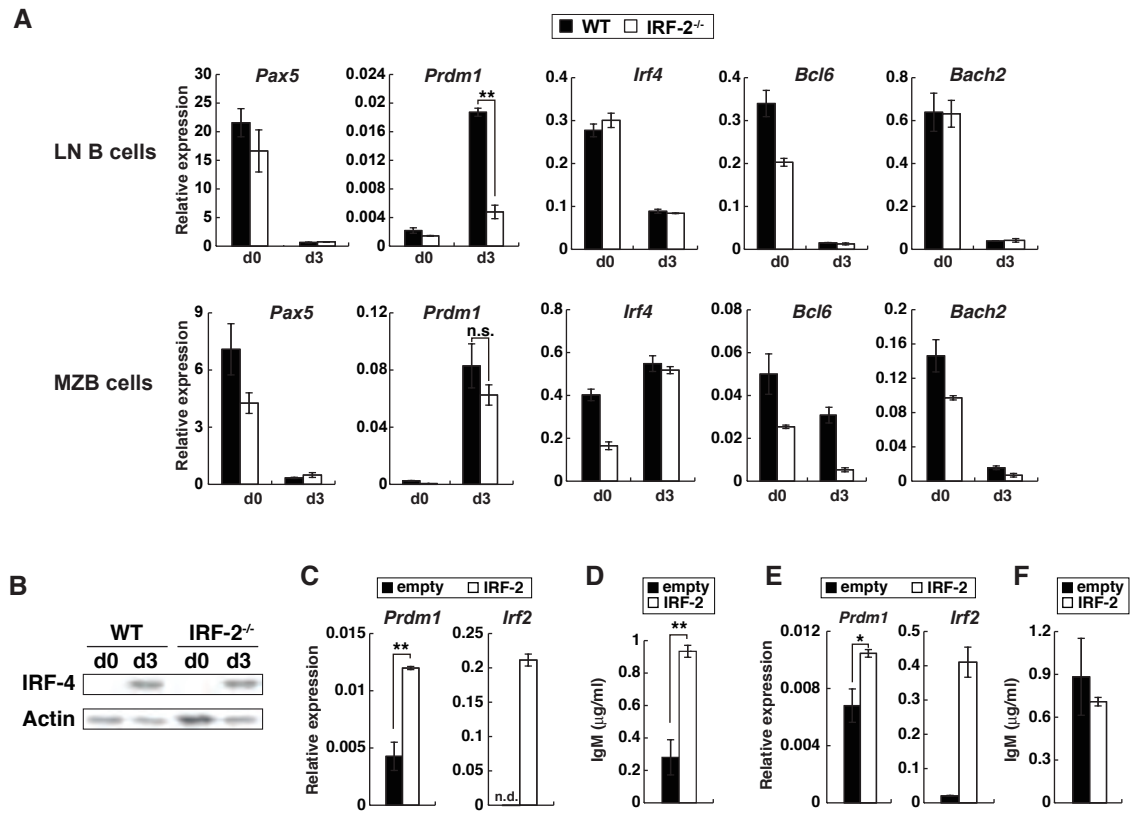
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Figure 3.



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Figure 4.



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Figure 5.

